10 Non-Target and Biodiversity Impacts in Soil

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Introduction

Possibly the most diverse of ecosystems can be found in soil. Soil contains many thousands of different species of bacteria, fungi, micro-, meso- and macrofauna, the numbers and activities of which are very temporally and spatially variable (see Box 10.1 for definitions of the different categories of soil organisms used in this chapter). The numerically dominant groups, the bacterial and fungal communities, can perform many functions and transformations such as the release and transformation of mineral nitrogen for plant growth, nitrogen fixation, plant-growth promotion, pathogen inhibition and phosphorus mobilization (van Elsas *et al.*, 1997). Soil meso- and macrofauna, such as earthworms, nematodes and arthropods, feed on living and dead plant tissues and play a vital part in soil nutrient cycling. For instance, they break the plant material into smaller pieces and redistribute it within the soil, making it better available for microbial activity and enhancing its incorporation into the soil organic matter. These biologically mediated processes are essential for providing the resources for sustainable plant growth and crop production and for the maintenance of all terrestrial ecosystems. Plants use the nutrients released during these transactions, and the soil organic matter and soil biota profoundly affect the soil structure and so water retention and drainage and the soil atmosphere.

Changes in the physiology, nutrient content or morphology of the crop plant caused by changes in the soil can strongly influence herbivore–plant interactions above ground, and therefore pest damage and/or the efficacy of the biocontrol community (see Chapters 6–9, this volume; Scheu, 2001).

 Environmental Risk Assessment of Genetically Modified Organisms: Vol. 2. Methodologies for Assessing Bt Cotton in Brazil (eds A. Hilbeck *et al.*)

Box 10.1. Definitions of categories of soil flora and fauna used in this chapter.

Soil microorganisms: 0.0002–0.002 mm in length and diameter.
Bacteria; fungi (yeasts).

Soil microfauna: length 0.004–0.2 mm, diameter < 0.1 mm.
e.g. slime moulds in the cellular phase (Acrasiomycetes); slime nets (Labyrinthulomycota); fungi; algae; amoebae; flagellates and ciliates (Protozoa); flatworms (Platyhelminthes).

Soil mesofauna: length 0.2–4 mm, diameter 0.1–2 mm.
e.g. plasmodial slime moulds (Myxomycetes); slime moulds in the pseudoplasmodia phase (Acrasiomycetes); mites (Acarina: Gamasina, Actineda and Oribatida); springtails (Collembola); nematodes (Nematoda); water bears (Tardigrada); rotifers (Rotatoria); enchytraeid worms (Oligochaeta); some flatworms (Platyhelminthes).

Soil macrofauna: length 4–80 mm, diameter 2–20 mm.
e.g. earthworms (Oligochaeta); pauropods (Pauropoda); symphylans (Symphyla); slugs and snails (Mollusca); millipedes (Diplopoda); centipedes (Chilopoda); woodlice (Isopoda); spiders (Arachnida); insect larvae and adults – some sucking bugs (Hemiptera: Cydnidae, Aphididae), some caterpillars or pupae (Lepidoptera), some fly larvae (Diptera), some beetle larvae and adults (Coleoptera: e.g. Carabidae, Scarabaeidae, Staphylinidae, Elateridae, Curculionidae, Silphidae, Cantharidae, Cerambycidae), termites (Isoptera), web-spinners (Embioptera), ants, etc. (Hymenoptera).

Soil megafauna: > 20 mm in diameter and length.
e.g. vertebrates, some large earthworms, some slugs and snails, some Carabid beetles.

There might also be an impact on the weed community in cotton that influences yield and insect populations, although control is generally intensive in most cotton systems (see Fontes *et al.*, Chapter 2, this volume). Impacts on soil functions may also negatively affect the sustainability of soil fertility and health in the longer term, even if they do not affect cotton production in the short term, due to compensatory effects of fertilizer and other farming inputs and operations.

The diversity of the soil biota can result in extremely complex food webs that are subject to a wide range of interactive influences. There are many different soil types, each essentially defined by texture. Texture is dependent on the relative proportions of mineral particles, sand, silt and clay, present. Soil also contains air spaces, water and organic matter. In such highly heterogeneous environments, physical conditions and chemical gradients change spatially and temporally, thus permitting a wide variety of biogeochemical processes to occur. Species and functional process diversity in plant–soil systems is immense and a vast range of compounds is produced, many of which

are then further transformed in other processes. These spatial and temporal variations over small to large scales make system predictions and modelling extremely difficult.

However, activity in the soil ecosystem is usually limited by the availability of nitrogen and energy from fixed carbon compounds derived from plant primary production, mainly plant-derived inputs from the roots, such as root exudates, cellular remains and root debris, and plant residues that fall on the soil surface (leaves, stems, flowers and fruit). Although microbial activity can occur in the bulk soil, it is much greater in the zone closest to the plant roots, the rhizosphere (Fig. 10.1). Inputs to the soil from the growing plant are greatest in the rhizosphere, and as these change with both the type and stage of growth of the plant, so do microbial functional dynamics. Microbial populations can increase by a hundred or a thousand times after plant material is added to soil. Plant species and genotype and soil physical conditions determine the amount and types of compounds entering the soil, and therefore indirectly drive the rates and types of biotic functioning. There can be large differences in the relative proportions of individual bacterial genera on different plant species and cultivars and in different soil types, and the influence of field site interacts with the influence of plant variety on the microbial community (Grayston *et al.*, 1998; Siciliano *et al.*, 1998; Dunfield and Germida, 2001). Soil ecosystem functional dynamics are also affected by other factors such as weather and cultivation techniques, but soil fertility depends primarily on microbial activity that is responsive to plant inputs, particularly in the plant-rooting zone.

As well as providing a basic energy source, these carbon inputs can also affect microbial function in more subtle ways, as they play a role in microbial interactions and signalling. Temporally variable interactions between general soil heterotrophs and specific groups of microorganisms, such as the autotrophic nitrifiers, occur in arable soils in response to additions of carbon and nitrogen. Transgenic insecticidal plants such as *Bacillus thuringiensis* (Bt) cotton produce and release relatively large amounts of a variety of novel proteins, including the active toxins. Microbial processes have been shown to be particularly responsive to protein substrates (Wheatley *et al.*, 1997, 2001). Changes in the root exudates of a transgenic plant may significantly alter the rhizosphere community associated with it (Brusetti *et al.*, 2004).

The authors looked at the three commercial Brazilian cotton-growing regions, the Midwest, the Meridian and the North-east. The three regions vary widely in both soil types and climate (see Fontes *et al.*, Chapter 2, this volume). In the North-east, cotton is grown at low altitudes in a xerophylic climate with relatively low irregular rainfall on relatively fertile soils. In contrast, in the Midwest, which is the major cotton-producing region, cotton is grown at altitudes from 400 m to 1000 m during humid summers with an average seasonal precipitation of 1100 mm, on relatively poor soils. In the Meridian region, cotton is grown at altitudes from 400 m to 700 m in a climate of hot and humid summers and cold and dry winters with average seasonal precipitation of between 900 mm and 1000 mm. Differences in crop-management

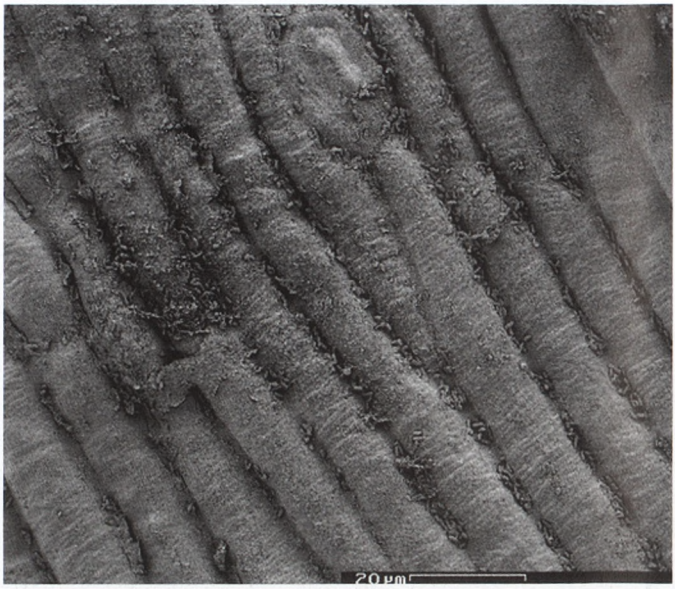

Fig. 10.1. Microcolonies of *Pantoea ananatis* strain A5M2 on the cotton root epidermis, 7 days after inoculation. Root colonization is often the limiting step in the use of rhizobacteria capable of producing indole-3-acetic acid in soil and promoting plant growth. Image taken in a field emission scanning electron microscope by Itamar Soares de Melo.

regimes between regions and between farmers will also influence soil dynamics and so affect the risk assessment. For instance, farmers in the Midwest use partial minimum tillage and plant millet after the cotton harvest. The next crop of cotton is then directly drilled into the millet residues, after desiccating herbicides have been applied. As a pest-control measure, they are obliged to chop up cotton crop residues before the following crop of millet. Disturbance of the soil has a tremendous effect on the functional dynamics of the soil organisms, mainly because of the redistribution and so enhanced availability of nutrients and organic matter that was previously spatially unavailable in soil aggregates, etc. Minimum-tillage systems disturb only the surface layers and so result in lower subsurface decomposition rates (Feng *et al.*, 2003). Therefore the crop-management system examined in the soil risk assessment is crucial to the outcome. We recommend that representative examples of all the alternative cropping systems are compared as multiple controls during the assessment of Bt cotton in Brazil.

Because of the vast complexity of the soil ecosystem, it would be both impractical and unreliable to study the system with rationales based on species lists. Although numerically there are far fewer functional properties, it is still impractical to measure all of them in the soil ecosystem, so choices of reliable parameters have to be made. In this chapter, we will consider the functional group 'soil ecosystem processes', and prioritize them for case-specific testing, based on the exposure pathway (association with plant residues and Bt toxin)

and the significance or possibility for an adverse effect (importance as an indicator of soil health and effect on cotton development). We will then identify adverse-effect scenarios for the highest-priority processes, formulate testable hypotheses and describe experiments that can test these hypotheses in the laboratory and field. See Box 5.1 in Hilbeck *et al.* (Chapter 5, this volume) for further detail.

This analysis does not constitute a complete risk assessment of the effects of Bt cotton on all soil ecosystems in Brazil. For example, as no significant commercial cropping occurs using 'organic farming' practices, that system is not considered. So consideration here is focused on determining a range of options that can be customized to a specific commercial scenario. A full characterization of risk would need to consider case-specific data from each of the Brazilian cotton-growing regions and agromanagement systems. Horizontal gene transfer between plant cells, bacteria and viruses was not considered in this chapter, but it should also be addressed in a full risk assessment of Bt cotton in Brazil.

Assessment of Input Routes and Possible Exposure Pathways in Soil

Bt proteins are present as active toxins in most of the cells of a Bt-transformed plant, and so are present in all plant residues, and may be released from root tissues (Fig. 10.2) into the surrounding soil. There are several reports of the long-term persistence of active Bt proteins in some soil types after the cultivation of Bt-transformed crops. As most of the activity in the plant–soil system occurs in the rhizosphere and is driven by plant inputs, the possibility that these Bt proteins may have a direct effect on all the biotic dynamics associated with plant production requires investigation before commercial release.

Input routes of Bt plant material and Bt toxin into soil

Bt plant material and Bt proteins can enter the soil through various potential routes, depending on the crop and environment:

1. The direct input of Bt proteins via root exudates and leachates from root injuries. This is a continual input of free, active Bt protein during the whole growing season, increasing in quantity and spatial extent as the root system grows.
2. Bt protein in the plant matrix of sloughed off root debris, e.g. root cap cells and root hairs. This is a continual input during the whole growing season, increasing in quantity and spatial extent as the root system grows.

The Cry1Ac protein in cotton with event 531 (INGARD® cotton) is expressed in the roots (OGTR, 2003). From 4 to 9 weeks after germination, Cry1Ac concentrations of between 1 μg/g and 43 μg/g dry weight of roots

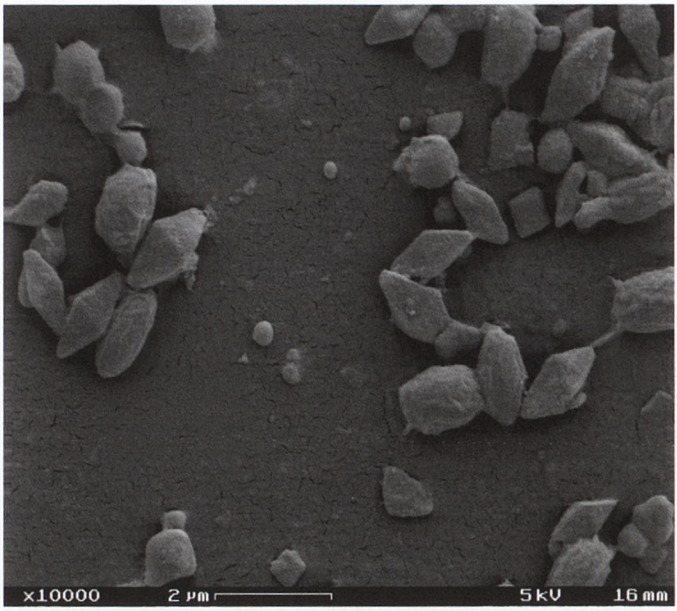

Fig. 10.2. Scanning electron micrograph of Bt toxin crystals and bacterial cells. Crystal produced by *Bacillus thuringiensis*. Image taken in a field emission scanning electron microscope LEO (Zeiss + Leica) at Embrapa Environment by Itamar Soares de Melo.

were found in Australia (Gupta *et al.*, 2002); the roots released Cry1Ac into soil during growth, although this was not quantified. Saxena *et al.* (2004) reported that Bt proteins are not exuded from Bt-cotton roots, though Bt maize, potato and rice roots do exude Bt. However, a certain amount of Bt protein will enter the soil throughout the growth period from decaying root tissues and leachates from root breakage. Bollgard cottons contain the same transgene and promoter as INGARD® cotton, so can be expected to express the protein in the roots similarly.

3. Input of Bt-expressing plant parts (leaves, squares, bolls, pollen) falling to the ground under the plants. This is a continual input during the whole growing season, increasing in quantity as the plants develop.
4. The input of plant residues (dead or alive, e.g. as seeds) remaining in the field after harvest. This is an annual or biannual input of relatively large amounts (depending on crop-management practices).

Sources of Bt-protein inputs and the pathways and processes by which they may have effects on soil ecosystems are summarized in Table 10.1. In this table, any item in the source column can be linked via any of the properties in the pathways and process columns to realize any of the outcomes in the effects column: e.g. pollen (source) may be ingested by fauna (pathway), degraded (process) and so decayed (effects).

Table 10.1. Effects pathways matrix.

Source	Pathway	Processes	Effects
Plant residues	Decomposition	Adsorption	Decay rate
Pollen	Protein release	Denaturation	Persistence
Root exudates	Faunal ingestion	Degradation	Bioactivity
Damaged tissues	DNA transfer	Plant uptake	Accumulation
Bt protein in soil		Elimination	
Transgene		Leaching	
		Run off	

These interaction routes between the Bt plant material or Bt proteins and the soil biota are strongly influenced by the tillage system. Under conventional tillage the plant litter will be mechanically incorporated into the soil, diluting the concentration of the protein but increasing the number of organisms exposed. Under zero tillage, crop residues are left concentrated on the soil surface and the only incorporation that occurs is through the action of soil macroinvertebrates.

Fate of the Bt plant material and the Bt proteins in soil

The fate of the plant materials containing Bt protein and the free Bt proteins in soil ecosystems depends on the rate of input and the rate of dissipation. For exposure and impact analysis of the incorporated and released Bt proteins on the soil ecosystem, it is essential to know how long they persist in the system.

Persistence – microbial or chemical degradation

Laboratory studies on the persistence of ground up lyophilized Bt cotton plant material in soil (in which the conditions for degradation were usually near optimal) have reported that the toxins were still detectable and retained their insecticidal activity when the experiments terminated after 28 days (Palm *et al.*, 1994; Donegan *et al.*, 1995), 120 days (Sims and Ream, 1997) and 140 days, at which point 25–30% of the Cry1Ac proteins produced by Bt-cotton leaf residues were still bound to the soil particles at pH 6.2 (Palm *et al.*, 1996) (see adsorption below). Even longer time periods have been reported using Bt maize residues in the laboratory (Saxena and Stotzky, 2000, 2001b; Hopkins and Gregorich, 2003) and in the field (Zwahlen *et al.*, 2003a,b). The general pattern is similar to that for other proteins and organic compounds in soil. Initially, a rapid decline of the soluble toxin concentration is observed, followed by a more gradual decline to low concentrations of the toxin that may then remain almost unchanged for several weeks and probably months (Palm *et al.*, 1996; Zwahlen *et al.*, 2003a). The persistence of Bt proteins in soil could therefore be long enough to be of concern through the growth cycle of the following crop. In the Midwest and North-east regions in particular, cotton is grown repeatedly on the same land year after year. It is

also possible that other Bt-transformed crops such as Bt maize will be grown in rotation with Bt cotton in future.

Decomposition rates are very dependant on soil pH, temperature and water availability, and vary with soil structure and texture as indicated, for instance, in Zwahlen *et al.* (2003a) and Donegan *et al.* (1995). Finer, clayey soils show, on average, slower decomposition rates and higher retention of organic matter than coarse, sandy soils. The recovery of the Bt protein from soil is also temperature and pH dependant (see also under adsorption below), and procedures used to extract Bt toxins from plant residues need modification to the experimental set-up before use (Palm *et al.*, 1994).

A field study on six fields, where Bt cotton had been grown for 3–6 years and the crop residues incorporated into the soil after harvest, found no detectable Cry1Ac toxin (by enzyme-linked immunosorbent assay (ELISA) and bioassays with *Heliothis virescens* on ground homogenized soil samples) in soil samples taken at 3 months after postharvest tillage (Head *et al.*, 2002). Three fields were sandy loam and three silt loam, and the experiment did not indicate differences between soil types. The authors take this as a demonstration that Bt protein from Bt cotton will not accumulate in the soil under temperate field conditions. Another study compared conventional-till and no-till cotton systems with Bt/glyphosate-tolerant cotton and glyphosate-tolerant cotton without Bt (Lachnicht *et al.*, 2004). They found no significant differences in decomposition between the two transgenic cotton cultivars, measured by change of weight and C:N content of buried litterbags. There was a significant difference between the tillage treatments.

Uptake by soil organisms or plants

Roots are known to reabsorb previously exuded organic compounds, a mechanism used in the uptake of insoluble cations. The uptake of Bt proteins from the soil into growing plants is therefore a possibility, but has not been investigated for cotton. One study investigated the uptake of Bt toxins from soils in which Bt maize had been grown (so containing Bt from root exudates, etc.), or soils in which Bt maize residue or purified Bt toxin was added. None of the test plants grown on these soils (maize, carrot, radish and turnip) took up any Bt proteins from the different soil types over 120 or 180 days. Also, isogenic maize did not take up the Bt toxin when grown in a hydroponic solution in which its Bt counterpart had been grown previously (Saxena and Stotzky, 2001a).

Soil fauna can contact Bt plant roots, residues and the Bt proteins still within the plant cells through their feeding activity as well as by physical contact with any Bt proteins bound to organic matter and clays in the soil. In addition, soil herbivores and detritivores may utilize the Bt protein as a novel food source, or they may sequester it and pass it on up the food chain. Trophic relationships in soil are very complex and require precise experimental design. Researchers found no detectable effects of Bt-cotton leaf tissue on two detritivores, the springtail *Folsomia candida* and the mite *Oppia nitens* (Yu *et al.*, 1997). However, it is not clear whether the organisms actually consumed the Bt material during the experiment, because they are both species that prefer-

ably consume fungi growing on decaying plant material (Seniczak and Stefaniak, 1981; Fountain and Hopkin, 2005). It has not yet been demonstrated whether or not fungi growing on decaying Bt plant material also contain the Bt toxins.

Adsorption of the Bt protein

The mobile Bt protein will be rapidly degraded by microbial degradation if it is not leached first (see below; Palm *et al.*, 1996; Saxena *et al.*, 1999; Saxena and Stotzky, 2000). However, laboratory experiments with microbially produced Bt toxin showed persistence and insecticidal activity at 234 days (Tapp and Stotzky, 1998). Bt toxins have been shown to bind to clay minerals and humic acids within a few hours of entering the soil and remain bound for long periods of time (Venkateswerlu and Stotzky, 1992; Tapp *et al.*, 1994; Tapp and Stotzky, 1995; Crecchio and Stotzky, 1998). Correspondingly, most (about 10% to 30%) of the Bt toxin can be recovered (washed out) from soils with low organic matter and high sand content, and least from soils high in clay content and organic matter (Palm *et al.*, 1994; Saxena and Stotzky, 2000). Adsorption is lower at pH much below or above 6. The Bt proteins only partially intercalate the clay mineral montmorillonite and do not intercalate kaolinite, so it can be assumed that they bind primarily on the external surface of the minerals (Tapp *et al.*, 1994; Saxena and Stotzky, 2000). The adsorbed Bt toxins retain their insecticidal activity (Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998; Tapp and Stotzky, 1998). Over 80% of the microorganisms in soil are adsorbed onto organic matter and clay minerals (Bruinsma *et al.*, 2002). The majority of microorganisms could therefore be in close proximity to the adsorbed Bt proteins.

Adsorption of the proteins to clays and humic acids in the soil will increase the greater the concentration of the protein entering the soil, but at high concentrations binding will level off at an equilibrium and more of the protein will remain mobile, indicating a saturation of the adsorption sites (Stotzky, 2000). When the proteins are bound to clay minerals or humic acids in the soil they are resistant to microbial degradation, therefore overall degradation rates are related to the rate of mobilization of the Bt protein (Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998).

Removal from soil (e.g. through leaching)

What is the persistence of the transgene product in the soil water and what is the likelihood of transgenic crop residues or the transgene product being transported in runoff from the transgenic field to adjacent fields or water bodies? A laboratory experiment was carried out using purified Bt toxin, using growing Bt maize exuding Bt from the roots and using Bt maize residues in soil columns containing differing quantities of clay minerals (montmorillonite and kaolinite; Saxena *et al.*, 2002). The Bt (Cry1Ab) protein was found in the leachates of all soil columns and not in the leachates of the respective isolines. The protein exhibited stronger binding and higher persistence, as well as remaining nearer the soil surface, in soil that contained higher clay concentrations (i.e. had a higher cation-exchange capacity and specific surface

area). This experiment indicates that there is a possibility that Bt proteins can be transported in water, either in surface waters by runoff and erosion or downwards.

Conclusions: input routes and possible exposure pathways

The Bt proteins will enter the soil inside both fresh and decaying cotton plant material and also as free protein in leachates from plant material. The soil fauna can therefore be simultaneously exposed to a complex combination of living and degrading plant material, free Bt proteins and Bt proteins bound to soil particles. Concentrations of Bt toxin in soil can fluctuate greatly with the highest concentrations occurring around decaying organic matter and roots. There is evidence that Bt toxins are present as long as the incorporated Bt plant residues are present, a 'time release' mechanism (Zwahlen *et al.*, 2003a). It is not clear whether this was tested in a Bt-cotton field study (Head *et al.*, 2002), as the cotton plant residue content of the samples was not stated, and so it is unclear if the bioassay *H. virescens* was actually exposed to any Bt plant material. It is also not clear whether or not the Bt toxins from Bt cotton will persist in the field outside of the plant material, bound to clays or humic acids, as has been shown for purified Bt in the laboratory and for Bt maize in the field (Saxena and Stotzky, 2000).

Soils in the Midwest, the major cotton-producing area, are mainly latosols or argisols (Fontes *et al.*, Chapter 2, this volume). As the clay component in argisols is moderate and moderate to high in latosols, it is possible that considerable amounts of Bt proteins may become bound in these soils.

Prioritization and Selection of Soil Processes

Soil ecological processes were considered in five main categories (Table 10.2):

- plant residues decomposition;
- biogeochemical nutrient cycling;
- plant/microorganism/meso- and macrofaunal interactions;
- cotton pests and diseases;
- role of biological activity in soil chemical and physical properties.

The significance of the ecological process, and therefore the significance of an adverse effect, was ranked by considering the importance of the process as an indicator of soil health and by considering how directly a variation in the process affects crop development or whether other ecosystem processes intervene to mitigate the effect of this variation. Direct effects involve a change to the process that directly affects crop development, and indirect/mediated effects are changes in the process that are mitigated by other ecosystem processes.

The exposure of the soil biota responsible for the process to cotton plant material (and proteins released from it) was considered by identifying the

Table 10.2. Ranking of soil processes in cotton-production areas in Brazil, main parameters.

Soil process	Associated biotic groups in cotton in Brazil	Importance as an indicator of soil health in cotton	How directly does the process affect cotton development?	Location of process in soil	Association with cotton plant material or released proteins	Expert ranking
(a) Plant residue decomposition						
Plant residue incorporation (biological)	Springtails, woodlice, ants, termites, epigeic and anecic earthworms, millipedes, beetles, molluscs, mites	High	Direct	Soil surface, upper soil layer (depth is specific to soil type and tillage system)	Direct (leaves, stalks, flowers, pollen, bracts, lint, seeds on soil surface)	1
Plant residue diminution (biological)	Springtails, woodlice, millipedes, ants, termites, anecic earthworms, mites, enchytraeid worms, symphylans, slugs and snails	High	Direct	Soil surface, upper soil layer (depth is specific to soil type and tillage system)	Direct (plant residues in the soil)	1
Organic matter decom-position (microbial)	Bacteria, fungi, slime moulds	High	Direct	Upper soil layer, particularly rhizosphere (depth is specific to soil type and tillage system)	Direct (plant residues in the soil)	1
Organic matter decomp-osition: cellulose breakdown (microbial)	Cellulytic bacteria and fungi	Medium	Semi-direct	Upper soil layer, particularly rhizosphere (depth is specific to soil type and tillage system)	Direct (plant residues in the soil)	2

Continued

Table 10.2. Ranking of soil processes in cotton-production areas in Brazil, main parameters – *cont'd.*

Soil process	Associated biotic groups in cotton in Brazil	Importance as an indicator of soil health in cotton	How directly does the process affect cotton development?	Location of process in soil	Association with cotton plant material or released proteins	Expert ranking
Organic matter decomp-osition: cellulose breakdown (meso- and macrofaunal)	Termites, oribatid mites, earthworms, slugs and snails	Medium	Semi-direct	Upper soil layer, particularly rhizosphere (depth is specific to soil type and tillage system)	Direct (plant residues in the soil)	2
(b) Biogeochemical nutrient cycling						
Ammonification (microbial)	Ammonifying bacteria	High	Semi-direct	Upper soil layer, particularly rhizosphere	Direct (plant residues in the soil)	1
Nitrification (microbial)	Nitrifying bacteria	High	Semi-direct	Aerobic soil layer	Direct (free Bt toxin in the soil) Indirect (NH_4^+ concentration)	1
Denitrification (microbial)	Denitrifying bacteria	Low	Semi-direct	Anaerobic soil layer/ conditions (e.g. waterlogging)	Direct (free Bt toxin in the soil) Indirect (NO_3^- concentration)	3
Nitrogen fixation (microbial)	Nitrogen-fixing bacteria and algae (e.g. *Rhizobia*)	Low	Indirect/ mediated	In aerobic, neutral pH soil layer – free living in soil, associated with grass roots, symbiotic on legume roots	Direct (free Bt toxin in the soil)	3

Phosphorus mobilization (fungal)	Mycorrhizal fungi on plant roots, mycorrhizal propagules	High	Indirect/ mediated	On cotton roots – rate is very dependent on fertilizer use – principally in poor soil Fungal ecology	Direct (association with plant roots)	2
Mobilization of micronutrients (fungal)	Mycorrhizae on plant roots	Medium	Semi-direct	On cotton roots Depends on plant nutritional status	Direct (association with plant roots)	2
(c) Plant/microbial/meso- and macrofaunal interactions						
Parasitism or commensalism in plant roots (micro-organisms)	Endophytic bacteria, endophytic fungi and endophytic nematodes	Medium	Semi-direct	In root plant tissue in between cells (phyllosphere)	Direct (root cells)	2
Parasitism or commensalism on plant roots and in rhizosphere (micro-organisms)	Rhizosphere bacteria, parasitic or symbiotic fungi (Mycorrhizae)	High	Indirect/ mediated	On root surface, and in rhizosphere (consumption of root exudates)	Direct (root cells)	2
Bitrophic feeding on plant roots (crop pests)	Nematodes, beetle larvae, termites, some symphylids, some endogeic earthworms, some springtails, some Dipteran larvae, root aphids, sucking bugs (Cydnidae)	High	Direct and mediated (through disease attack)	On roots, in rhizosphere	Direct (plant roots)	1

Continued

Table 10.2. Ranking of soil processes in cotton-production areas in Brazil, main parameters – *cont'd.*

Soil process	Associated biotic groups in cotton in Brazil	Importance as an indicator of soil health in cotton	How directly does the process affect cotton development?	Location of process in soil	Association with cotton plant material or released proteins	Expert ranking
Saprophytic feeding on plant residues (overlap with decomposition function above)	Bacteria, fungi, slime moulds, water bears, enchytraeid worms, pauropods, symphylans, mites, slugs and snails, woodlice, millipedes	Medium	Indirect/ mediated	Soil surface, upper soil layer (depth is specific to soil type and tillage system)	Direct (plant residues)	3
Feeding on soil fungi (functions: keeping colony in young state, dispersal of fungi in soil, etc.)	Slime moulds, springtails, fungiphagal bacteria, symphylans, pauropods, nematodes, enchytraeids, woodlice, millipedes, Dipteran larvae	Medium/low	Semi-direct	Rhizosphere and detritus	Possible tritrophic (via residue and roots/root exudate-consuming fungi)	3
Feeding on other microbial flora and fauna (functions: keeping colonies in young growing state, dispersal of bacteria in soil, etc.)	Protozoa, slime moulds, symphylids, pauropods, springtails, nematodes, some water bears, rotifers, endogeic earthworms	Medium	Semi-direct	On plant roots, in rhizosphere, in soil free-living, in soil bound (bacteria)	Possible tritrophic (via residue and root/root exudate-consuming fungi and bacteria)	2

Predation on soil meso- and macro-fauna	Carabid beetles, Staphylinid beetles, spiders, ants, centipedes some Dipteran larvae, some slugs, some gamasid and actinid mites and some spring-tails (eating nematodes)	High	Semi-direct (trophically mediated)	Soil surface, upper soil layer	Possible tritrophic or multitrophic (via residue and root-consuming meso- and macro-fauna)	2
(d) Cotton pests and diseases						
Disease transmission	• Fusarium wilt (*Fusarium oxysporium* f. sp. *vasinfectum*) spores and hyphae	High	Semi-direct	• On roots and plant residues in soil	Direct (roots)	1
	• *Verticillium dahliae* spores			• Hyphae and spores in plant residues on soil surface	Direct (plant residues on soi surface)	
	• Other fungal hyphae and spores e.g. *Rhizoctonia solani*			• In residues on soil surface		
	• Bacterial blight			• In residues in soil	Direct (plant residues in soil)	
Disease suppression (by grazing or antagonism) (e.g. Lartey *et al.*, 1994)	Bacteria e.g. *Bacillus thuringiensis*, *Pseudomonas fluorescens*	Low	Indirect/ mediated	Rhizosphere and roots	Direct (free Bt protein)	2
	Antibacterial and parasitic fungi e.g. *Trichoderma viride*				Direct (root exudates on plant roots)	
	Fungal feeders (see above)				Indirect (in soil)	
Predation on crop pests in soil	Predators of root feeders, see predation above	Medium/ high?	Semi-direct	On cotton plant roots, rhizosphere, in upper soil layer	Possible tritrophic – via prey exposed to roots, plant residues	2

Continued

Table 10.2. Ranking of soil processes in cotton-production areas in Brazil, main parameters – *cont'd.*

Soil process	Associated biotic groups in cotton in Brazil	Importance as an indicator of soil health in cotton	How directly does the process affect cotton development?	Location of process in soil	Association with cotton plant material or released proteins	Expert ranking
(e) Influence of biological activity on soil chemical and physical properties						
Binding/storage of nutrients (production of humic substances)	Calcitrant (humic) soil organic matter (producers: bacteria, fungi, enchytraeid worms, slugs and snail mucus)	High	Indirect/ mediated	Strongly dependent on soil type, pH, crop management (tillage, crop rotation, etc.)	Indirect via rate of production of calcitrant soil organic matter	2
Soil aggregate formation (soil aeration, structure and stability, resistance to erosion)	Soil organic matter and carbon compounds (polysaccharides) from decomposition: fungal hyphae (including Mycorrhizae), bacterial colonies, enchytraeid worms, slugs, snail and earthworm mucus, etc.	High	Indirect/ mediated	Strongly dependent on soil type, pH, crop management (tillage, crop rotation, etc.)	Indirect via impacts on soil organic matter production, impacts on fungi, bacteria, earthworms, etc.	2
Water movement in the soil (drainage)	Pores formed from earthworm burrows, fungal hyphae, etc. Soil aggregate structure	High	Indirect/ mediated	Strongly dependent on soil type, pH, crop management (tillage, crop rotation, etc.)	Indirect via impacts on micro and macro-organisms and impacts on soil aggregate structure	2
Water retention in the soil (soil moisture content)	Soil aggregates and spaces formed from soil organic matter	High	Indirect/ mediated	Strongly dependent on soil type, pH, crop management (tillage, crop rotation, etc.)	Indirect via impacts on soil organic matter production and soil aggregate structure	2

location of the functional activity in the soil, i.e. possible direct-exposure routes to cotton plant material and proteins or whether an effect might occur through an indirect impact. Considering both significance and exposure in cotton systems in Brazil, the soil processes were ranked on a 1, 2, 3 basis for each process, 1 being of the greatest priority and 3 the lowest. The highest-priority processes have direct importance as indicators of soil health, as the functional biota are closely associated with living or dead crop plant material or exudates and an adverse effect on the process would likely have a direct impact on crop development and yield stability.

One concern could be the possibility of an alteration in the rate, timing or magnitude of soil organic matter decomposition that could have consequences for soil health and fertility, including a change in energy and nutrient supply for other microbial processes, and for plant growth. Similarly, the stability of soil aggregate structure may be affected with consequences for root development and water release and holding capacities. The consequences of any deleterious effects on residue decomposition are so great that this is ranked 1. As nitrogen-cycling dynamics in agricultural environments are known to be entirely dependent on the quality and quantity of plant inputs, these processes might be directly affected by input changes, particularly with accompanying potential toxicity effects, and so the two important steps in nitrogen cycling in high to medium input cotton production (ammonification and nitrification) are ranked 1. A possible impact on disease transmission might have significant consequences because of the importance of soil-transmitted fungal diseases in limiting cotton production in Brazil (see Fontes *et al.*, Chapter 2, this volume), and, although it was considered to be likely to appear over a relatively longer time scale, was ranked 1.

The selection process could be further developed by considering criteria such as the vulnerability of the process to change in the soil, the amount of redundancy in the soil biota responsible for the process and the experimental accessibility (Kowalchuk *et al.*, 2003).

Selection matrix for soil macrofauna

A functional assemblage approach was also applied to the meso- and macrofauna involved in decomposition using a selection matrix (Table 10.3). The advantage of this approach is that it enables a transparent selection process to be defined, despite the paucity of species-specific information available on soil macroorganisms in Brazilian cotton cultivation. This approach can be modified and refined as information becomes available. We did not consider vertebrates in cotton fields in Brazil.

The macrofauna was divided into three functional groups:

- decomposers;
- root feeders;
- disseminators of residues.

For reasons stated before, the priority concern is that any impact on soil macroorganism functions may slow down the rate of residue breakdown and

Table 10.3. Macrofaunal rankings for decomposers.

Functional group	Taxa	Other functions	Possible indicator groups	State of knowledge[a]	Priority[b]
Decomposers (litter breakdown)	Collembola, mites, Isopoda, Diplopoda, Symphyla, beetles, ants, etc.	• Many are also disseminators of residues	Millipedes	1	1
Root feeders	Nematodes (pathogenic), insect larvae	• Damage to crop plant • Facilitators of entry for disease pathogens	Nematodes	1	2
Disseminators of residues	Earthworms, other soil-burrowing species; Collembola, ants; beetles, (many species are multifunctional)		Earthworms	1	1 and 2 (earthworms rank 1)

[a]1, fairly good; 2, partial; 3, not satisfactory.
[b]1, high priority; 2, intermediate priority; 3, low priority.

incorporation into soil organic matter, and as this drives the rate of organic matter decomposition by the microbial community in soil, may have consequences for soil health and plant growth. The macroinvertebrate disseminator group was therefore ranked 1.

The soil meso- and macrofauna fulfil many other important functions in soil such as dispersers of beneficial microbial populations in the rhizosphere, as dispersers of saprophytic fungi (Rantalainen *et al.*, 2004), as seed dispersers and as predators of crop pests. In addition, epigeic macroinvertebrates can be an essential food source for generalist predators above ground (e.g. for Carabid beetles, Staphylinid beetles and spiders (Ekschmidt *et al.*, 1997), as well as for vertebrates such as birds or shrews). This can be particularly important in between crops or at the start of the crop-growing season (Settle *et al.*, 1996).

The analysis of the soil meso- and macrofauna in this chapter is incomplete as little is known now about the diversity present in Brazilian cotton fields. To encourage knowledge gathering in this area, we propose a starting model for this important grouping that can be refined and developed as further information becomes available. In this chapter, earthworms are used as an example of the disseminator group. Earthworms perform several functions, such as residue distributors and incorporators, assisting in decomposition, and are abundant and diverse. Site- and region-specific information on their relative significance and species diversity is required for a full analysis.

Identification of Adverse-Effect Scenarios and Testing Hypotheses

In this section, analysis of input routes and exposure pathways, and the results of the soil ecological-process prioritization, are used to develop adverse-effect scenarios for the soil ecosystem, and testing hypotheses to test the scenarios. In the final section of the chapter, specific experimental protocols are described to provide definitive scientific evidence that any potential effect or effect pathway is likely to occur or not.

Persistence in the soil

The first requirement for the verification of exposure routes is the assessment of the amounts of Bt proteins introduced into the soil ecosystem during and after the cultivation of Bt cotton, and their persistence. As the Bt proteins are in the active form there may be an enlarged target range that might adversely affect both micro- and macrofaunal organisms, with subsequent detrimental effects on functional dynamics in the soil ecosystem.

Verification of exposure pathway: how long will Bt proteins in plant residues and Bt proteins released into the soil from such plant residues and from the roots persist in the soil, and associated groundwater? Do they persist to the next cropping season?

Microbial (bacterial and fungal) decomposition of organic matter, and the coincident nitrogen-transformation processes

A vast array of microorganisms and higher trophic groups of organisms such as the micro- and mesofauna interact in the incorporation and breakdown of plant material in soil ecosystems. A study of the impact of leaf material from three Bt-cotton lines found no differences in the numbers of protozoa, but did find changes in culturable bacterial diversity, and significantly greater increases in culturable bacterial and fungal population levels with the transgenic material compared to the parental line in the 2 weeks after the start of the experiment (Donegan *et al.*, 1995). At the end of the experiments (28 or 56 days), these changes were no longer observed. This suggests that the transgenic plants may have decomposed faster than the parental plants and thus more rapidly provided nutrients for microbial growth, though decomposition was not measured. On two sampling occasions there was significantly greater utilization of asparagine, aspartic acid and glutamic acid in soil with material from the two transgenic lines compared to the parental line. These substrates are important intermediates in nitrogen-assimilation reactions. Because the changes were only observed for two of the transgenic lines and not the third, and not for the purified toxin, the authors conclude that they might be due to unintended changes in the physiology of these transgenic lines rather than directly due to the Bt toxins. Further investigations are required to determine what changes might occur with repeated incorporations. Studies on other transgenic plants have also found differences in microbial communities associated with the plants at the senescence growth stage, indicating an association with the decomposer community (Lottmann *et al.*, 1999, 2000; Lukow *et al.*, 2000).

Transforming a plant can influence its physiology in other than the specifically designed effect, either through pleiotropic effects of the transgene (Grossi *et al.*, Chapter 4, this volume), or metabolic effects of the transgenic protein, or changes arising from the transformation process (e.g. tissue culture) and subsequent breeding (Andow *et al.*, Chapter 1, this volume). Studies with other transgenic plant residues in soil have shown differences in the nutritive quality of the transgenic plant for soil microbial and macrofaunal communities, compared to the control or other varieties (Donegan *et al.*, 1997; Escher *et al.*, 2000; Saxena and Stotzky, 2001b,c). Such differences may have significant impacts on plant residue-degradation rates, and the non-transformed parental or near-isoline may not provide a completely satisfactory control for this reason (Andow and Hilbeck, 2004). Therefore, any comparative experiments may not only be assessing effects of the toxins but also changes in the organic constituents resulting from these effects on the plants' physiology. We recommend examination and comparison of the nutritive qualities of the transgenic and control plants (e.g. cellulose and lignin content, C–N ratio) prior to carrying out degradation experiments. See also earlier discussion (section on persistence) for the influence of environmental conditions (pH, temperature, water) and soil type on decomposition.

Adverse-effect scenario: The functioning of soil ecosystems is dependent on the breakdown of plant residues to provide energy. Microbial functional

dynamics are dependent on the quality and constituents of this input. During decomposition, the nitrogen-containing compounds required for many other microbial functions are released. So these plant–microbe relations have consequences for soil fertility and crop nutrition. As the patterns of such inputs are specific to the crop variety, it can be anticipated that microbial dynamics under Bt cotton plants will be directly affected by these plants. A change in degradation patterns in the soil may result in a disruption or asynchrony of energy and nutrient supply in relation to the demands of the crop, and so requires quantification.

Testable hypothesis 1: The rate, timing or magnitude of the decomposition of Bt-cotton plant residues will be altered compared to decomposition rates of conventional cotton, due to impacts of the residues on microbial activity.

Nitrogen cycling: ammonification

The recycling of inorganic nitrogen from plant residues for further crop production is a vital function of the soil ecosystem. These transformations require the interaction of a vast array of microorganisms and higher trophic groups, especially in the first step of ammonification. Ammonification, the conversion of organic-nitrogen to ammonium-nitrogen, is closely allied to decomposition.

Adverse-effect scenario: Ammonifying bacteria will come into direct contact with Bt toxins in the rhizosphere and around decomposing cotton plant residues. These toxins may affect their activity and therefore affect the rate of ammonification in the rhizosphere and soil near to the cotton plant, with further consequences for nitrification rates, decreasing the availability of nitrogen for the cotton and subsequent crops grown in the field.

Testable hypothesis 2: Ammonification rates will be reduced in soils containing Bt-cotton residues, with consequential effects on soil fertility and crop production.

Nitrogen cycling: nitrification

Nitrification is the next step in the N-cycle, in which the immobile ammonium form is converted to the mobile nitrate form that is preferentially taken up by plants. The process involves two bacterially mediated steps: first the oxidization of ammonia to nitrite and then the oxidization of this nitrite to nitrate. Nitrification is often a rate-limiting step in the nitrogen cycle and influences nitrogen availability to plants. It can decrease soil pH and lead to leaching of nitrogen from soil ecosystems, and is also the first step in the loss of fixed nitrogen from soil via denitrification. Denitrification can also lead to a significant loss of nitrogen in systems receiving nitrogen fertilizer (Bruinsma *et al.*, 2002), as does much of the cotton production in Brazil. Due to their relatively low redundancy, their sensitivity and their influence on an important nutrient cycle, nitrifying bacteria could be suitable indicators of soil health (Kowalchuk and Stephen, 2001; Bruinsma *et al.*, 2002).

To date, all known ammonia-oxidizing bacteria (AOB) in soil belong to a narrow clade within the β-subclass of the protoebacteria consisting of two genera, *Nitrosospira* (containing former genus designations *Nitrosolobus* and *Nitrosovibrio*) and *Nitromonas*. Although *Nitromonas europae* is the best-characterized species of AOB, probably due to the relative ease with which it can be recovered in pure culture, numerous studies have suggested that *Nitrosospira* species are dominant in terrestrial ecosystems (Kowalchuk and Stephen, 2001; Wheatley *et al.*, 2003). Some AOB groups may be indicative for specific environmental conditions.

Nitrite oxidization, the second step towards nitrate formation, is spread across several bacterial groups, making comprehensive molecular studies of this group more difficult. The step may be more susceptible to stress conditions, as nitrite can accumulate under various stress conditions (Bollag and Kurek, 1980), but ammonia oxidation is thought to be the rate-limiting step in nitrogen turnover in most terrestrial ecosystems (Prosser, 1989).

Adverse-effect scenario: Nitrifying bacteria need an aerobic environment, and are frequently found as colonies on the surface of soil aggregates. Bt toxins released from plant residues and roots will be adsorbed onto these same particles. This proximity could have consequences for the rates of nitrate formation, specifically ammonia oxidization, in soils cultivating Bt plants. Previous works (Wheatley *et al.*, 2001; Mendum and Hirsch, 2002) have shown that nitrification rates are particularly susceptible to changes in carbon inputs, particularly proteins.

Testable hypothesis 3: Inorganic nitrogen-transformation rates will be reduced in soils with Bt-cotton residues, with consequent effects on soil fertility and subsequent crop production.

Role of macroorganisms in decomposition of organic matter

Although ultimately organic matter decomposition is effected by microorganisms, the dynamics of this process are greatly increased by the activities of many macrofauna in many ecological niches. Although little information is available on the macrofaunal diversity of the cotton-cultivation areas in Brazil, it can be assumed that they also play an equally important role there. We propose initial basal studies to validate an indicator model to confirm this. This is based on examining one of the macrofaunal functional groups described previously: the disseminators. In a worst-case scenario, other groups can be added to complement the picture.

We suggest the use of earthworms as a model example for the examination of any possible effects of Bt-cotton cultivation on macrofaunal activity. Zwahlen *et al.* (2003b) reported no lethal effects of Bt maize litter on immature and mature *Lumbricus terrestris*, but a significant weight loss compared to worms eating litter of the non-transformed isoline in temperate maize production. The earthworms excrete the Bt toxin in a concentrated form in their casts. Casts from Bt maize-fed earthworms were found to be toxic to the Lepidopteran tobacco hornworm (*Manduca sexta*) (Saxena and Stotzky, 2001b).

Adverse-effect scenario: Earthworms may be sublethally affected by the Bt protein in the residues and soil they consume, and so their roles as incorporators and disseminators of plant material in the soil might be affected, which may have a slowing effect on the rate of decomposition of organic matter in the soil of Bt-cotton fields. They may also cause tritrophic effects on their predators from Bt in their guts, and on detritivores via the Bt toxin in their casts.

Impacts on residue-eating macroorganisms in soil will be dependent on the nutritional characteristics of the transgenic plant compared to the control, as mentioned above; therefore this should be taken into account. As well as any immediate lethal effects on macrofaunal members of the soil community, long-term sublethal effects possibly limiting functioning need to be considered. We recommend that other components of the soil macro- and mesofauna are also assessed.

Testable hypothesis 4: Macrofaunal activity and interactions (e.g. earthworms) will be adversely affected with a resultant decrease in plant-residue diminution and incorporation rates.

Cotton pest damage and disease transmission in soil

Most of the economically significant pests of cotton in Brazil are not soil dwellers at any life stage (see Sujii *et al.*, Chapter 6, this volume). Some weevil larvae are root borers (e.g. *Chalcodermus niger* (Hustache) and *Eutinobothrus brasiliensis* (Hambleton) [Coleoptera: Curculionidae]) and some Hemipteran bugs are root suckers (e.g. *Atarsocoris brachiariae* (Becker) and *Scaptocoris castanea* (Perty) [Hemiptera: Cydnidae]). A few Lepidopteran pests pupate in the soil, but as this is a non-feeding stage it is expected that there will be no susceptibility to any Bt proteins present in the soil. Contrastingly, there are several significant soil-borne fungal diseases of cotton (see Fontes *et al.*, Chapter 2, this volume). For example, *Fusarium oxysporum* that enters cotton plants through damage caused by the root-knot nematode *Meloidogyne incognita*, and is most severe in sandy soils, and *Verticillium dahliae*, which is most common in neutral to alkaline silt and clay soils of arid regions, particularly in irrigated cotton. Most other fungal diseases are transmitted to the growing cotton plant from infected plant residues via wind, water or insects, e.g. *Rhizoctonia solani*. Bacterial blight, caused by the bacterium *Xanthomonas axonopodis* pv. *malvacearum,* which causes damage from the seedling stage onwards, is carried over in infested seed and in crop residues on the soil surface.

Adverse-effect scenario: Disease-causing fungi or bacteria may be directly affected by the Bt toxin inside plant residues on which they are actively growing or resting as a dormant phase, or they may be indirectly affected by Bt-cotton cultivation due to changes in rates of input and degradation of plant residues. They may be able to increase their survival rate, which could increase infection rates of Bt cotton and negatively impact yields, or they may have reduced survival rates, which could positively influence Bt-cotton cultivation by decreasing the incidence of fungal and bacterial disease.

Testable hypothesis 5: Inputs from Bt cotton will alter the survival and efficacy of soil-borne fungal and bacterial diseases of cotton, compared to the influence of the non-transformed isoline.

Microbial biodiversity in soil

Studies on a range of different transgenic crops and traits have found differences in microbial and fungal community structure in the transgenic plant rhizosphere, compared to the non-transgenic control (for a review see Dunfield and Germida, 2004). These studies have used recently developed methods for the direct assessment of the degree of structural diversity in microbial communities. These molecular techniques can describe microbial populations as a whole, or in constituent parts (e.g. fungi), or functional groups (e.g. nitrifiers), depending on the choice of primers. Variable regions of 16S ribosomal genes are amplified from environmental DNA extracts by polymerase chain reaction (PCR), using primers specifically targeted at conserved regions in the bacterial genome; the primer set used determines the specificity. It is recommendable that the primers are tested and, if necessary, new primers developed for each transgenic trait and cultivar to be tested. The products can then be separated into different DNA bands on a gel by differential gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) (Muyzer and Smalla, 1998). A similar technique is terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997; Lukow *et al.*, 2000; Osborn *et al.*, 2000; Blackwood *et al.*, 2003).

Such methods overcome some of the limitations inherent in using culture techniques for identification and population descriptions, namely that only a small fraction of the microbial community can be successfully cultured in the laboratory and these may not necessarily be the most common components in that population (Hugenholtz *et al.*, 1998). Investigations of whole microbial population DNA by DGGE have shown changes in the whole soil population profile during the crop-growing season and under different cultivated crops (Smalla *et al.*, 2001; Pennanen *et al.*, 2003). This is shown by changes in the DGGE profiles of bands relating to taxonomic or functional groups of microbes in the population (Fig. 10.3). The methods are not quantitative, but the presence or absence of bands shows an appearance or disappearance of a group, and differences in the intensity of bands indicate that bacterial numbers are changing even if the group is still present. The banding patterns can be compared between the transgenic plant and non-transgenic controls, taking care to compare the same field sites and stages of crop growth. If comparing different soils, field sites and stages of crop growth, due allowance must be given to the amount of variation this causes compared to what the magnitude of a possible effect might be. It is to be anticipated that such protocols will show differences between different plants and also between the same plant when cultivated in different soils or under a different management system. However, data from experimentation with non-transformed plants

show that these are transient changes that are dynamically responsive to the conditions prevailing at the time of sampling (Gomes *et al.*, 2001, 2003).

Additional information can be obtained from community-level physiological profiling (CLPP) using Biolog plates (Griffiths *et al.*, 2000; Buyer *et al.*, 2001). The Biolog method is based on the growth on a variety of substrates, so gives some information on both species and functional diversity. However, as the functions detected in the plates can be performed by one of several species, any loss of species biodiversity will not be seen. Phospholipids fatty acid profiles (PLFA) (Blackwood and Buyer, 2004) can also be used to examine the phenotypic composition of communities. As different subsets of the microbial community have differing PLFA profiles, changes in the ratios between them can be determined. Preferably, such methods should be targeted to specific known functional groups or organisms, and not used for general community analysis.

Changes in microbial community structure may have adverse effects on functional dynamics, especially those associated with decomposition and nutrient cycling. Many soil macrofauna feed on the microbial community,

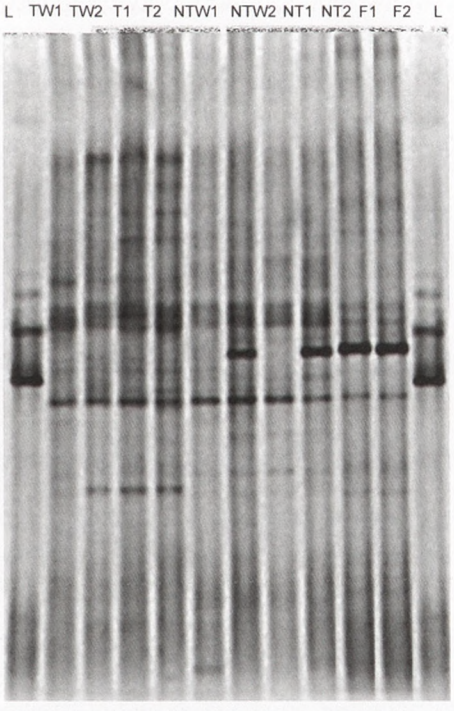

Fig. 10.3. DGGE banding pattern of *rpo*B PCR amplification of soil samples (0–5 cm depth). L = marker (from top to bottom *Staphylococcus aureus* MB, *Bacillus subtilis* IS 75, *Escherichia coli* HB101); TW = tillage with winter cover crop; T = tillage without winter cover crop; NTW = no-tillage with winter cover crop; NT = no-tillage without winter cover crop; F = native forest; 1 = first sampling; 2 = second sampling. Image from Raquel Peixoto, MSc dissertation, Federal University of Rio de Janeiro.

and so soil macroinvertebrates may also be adversely affected if microbial diversity is reduced. It is important to consider that microbial communities with similar structures as determined by these methods may still have ecologically significant differences in species composition, as the methods are not generally sensitive to changes in community structure at the individual strain or species level. The methods may only assess any changes in the numerically dominant portions of the microbial populations in soil. Minority microbial populations may not be represented as the template DNAs from these populations will form only a small fraction of that from the total community, and so are not adequately amplified to levels that can be detected above the background (Liu *et al.*, 1997). Therefore, these methods may not provide definitive answers to specific adverse-effect hypotheses, but indicate changes in community structure that may or may not have consequences on soil functional dynamics.

Changes in microbial communities are not permanent, as further changes in the bacterial community structure will occur if another type of crop replaces that grown before, or if the soil remains fallow. However, exposure of the microbial community will continue whilst the transgenic crop residues remain in the soil, and this may be the case over a relatively long time period after the crop is harvested (see persistence above), particularly as cotton is grown repeatedly year after year in many of the cotton-producing areas in Brazil.

Prerelease Experimental Designs for Potential Effects or Effect Pathways

Experimental protocols were designed to provide scientific evidence of the magnitude and duration of any effects of the Bt toxin on the soil ecosystem. These encompass appropriate spatial and temporal scales, sufficient replication, samples and relevant controls so that statistical power is not an issue for interpretation of the results. Because such a wide range of soil ecosystem processes might be affected in some way by changes in any inputs to the system, these experimental processes should be begun early in the development of any transgenic plant at the stage where the plant is being characterized in the laboratory, before field release (Andow *et al.*, Chapter 1, this volume). Some experiments can be carried out using microbially produced toxin, before the transgenic plant material is available. However, caution is urged when comparing the results, as the addition of plant material to soil has a strongly stimulatory effect on microbial activity, which the purified protein will not have (Donegan *et al.*, 1995; Palm *et al.*, 1996), and results may not be comparable for other reasons. It is important to note that on some occasions significant and contradictory differences have been found between experiments with the purified Bt toxin, even if this is identical to the Bt toxin that is produced in the transformed plant, and experiments using Bt plant material. Further experiments can be carried out using transgenic plants and soils in the greenhouse. Protocols to address the hypotheses in this chapter are described below (see Birch *et al.*, 2004, for further experimental protocols for laboratory and greenhouse).

We recommend that representative examples of all the alternate systems (chemical control, biological control and transgenic) are compared as multiple controls for the assessment of Bt cotton in Brazil. We also recommend that several local non-transgenic cotton cultivars are used as controls, as well as the non-transgenic isoline, provided it is available. The Bt-cotton variety to be tested should be locally adapted, with sufficient non-transgenic host plant resistance to the most damaging diseases and pests in each region. If a non-locally adapted variety is the only one available, it is necessary to consider that the plants may be disproportionately affected by disease and pest damage in comparison with the local non-transgenic varieties and this may influence the results.

It is important that investigations of the impact of transgenic plants sample at various points in the crop-growing season, so as to cover the variation in the microbial community as the crop develops (Smalla *et al.*, 2001; Heuer *et al.*, 2002; Dunfield and Germida, 2003). The spatial heterogeneity of the soil ecosystem is also an important issue (e.g. Lukow *et al.*, 2000). Although the majority of cotton cultivation in Brazil is on clay soils, a true representation of the other soil types, particularly those with low clay or high organic matter content, must also be examined.

The authors propose that the following experimental protocols are all carried out with material from the same series of incubated samples. As well as reducing the workload this will remove, or at least reduce, variations in the sample material caused by the inherent heterogeneity of the soils. Soils will be collected from cotton fields at:

1. Three sites in the Midwest region.
2. One site in the North-east region.
3. Two sites in the Meridian region.

This gives a total of six different representative soils from the three regions. Collect soil from the rooting zone or the equivalent in the field, from beneath cotton plants and unplanted areas. Take from several positions in the field, at least five points. Pass through a 4 mm sieve, bulk, homogenize and transport to the laboratories.

Amendments: Determine the amounts of residues entering the system at the sites from which the soils were taken. Then add Bt and non-Bt residues, from isolines, to the soils at the following rates:

1. 0.0 × field rate
2. 0.5 × field rate
3. 1.0 × field rate
4. 2.0 × field rate
5. 3.0 × field rate

The field rate is determined by measuring the amount of cotton crop residue in the field; it is anticipated that this will be about 8 t/ha (J.O. Siqueira, Brasília, Brazil, June 2004, unpublished data). Residues are placed on the soil surface and also mixed with the soil. Standard incubation conditions should be used for all six soils: adjust soil moisture content to 70% of soil pore space, incubate at

a diurnal cycle of 25°C (12 h) and 17°C (12 h), use 5–10 kg of soil (dry weight) in a lysimeter, incubate for at least 360 days (Saxena and Stotzky, 2003). Subsamples can be taken at 0, 7, 15, 30, 60, 90, 120, 150, 180 and 360 days, depending on the requirements of specific analyses. Sample size is 5% of the total soil. At the same time, collect the leachates from the lysimeters, measure and record the volume. Incubate five replicates of each treatment.

Verification of exposure pathway: How long will Bt proteins in plant residues and Bt proteins released into the soil from plant residues and from the roots persist in the soil? Do they persist to the next cropping season?

Experiment: concentration and persistence of Bt proteins in soil, laboratory determinations

Monitor Bt protein concentrations in the incubated soils and associated leachates over time, as described above. Incubations should be continued until all the Bt proteins have apparently disappeared. Five replicates are required, using both soils that have previously cultivated either Bt cotton or the isoline and unamended soils of both types to be used as controls. Bt protein concentrations can be evaluated by several methods:

1. Bt protein presence using lateral flow quickstix. These give a qualitative or sometimes semi-quantitative result (Palm *et al.*, 1994, 1996; Head *et al.*, 2002; Herman *et al.*, 2002; Saxena and Stotzky, 2003).
2. Bt protein concentrations by ELISA (Zwahlen *et al.*, 2003a).
3. Bt protein identification by capillary electrophoresis.
4. Bt protein toxicity by bioassays; grow a target Lepidopteran larva that is highly susceptible to the Cry protein being examined, such as *M. sexta* for Cry1Ac, in the test soils at various time points during the soil incubations (e.g. Saxena *et al.*, 2004; Flores *et al.*, 2005).

Measured endpoints: Measure rates of both input and decline of the Bt proteins to estimate persistence. Determine the median lethal dose (LD_{50}) (Saxena and Stotzky, 2001a).

Testable hypothesis 1: The rate, timing or magnitude of the decomposition of Bt-cotton plant residues will be altered compared to decomposition rates of conventional cotton, due to impacts of the residues on microbial activity.

Experiment: assessment of microbial activity and decomposition rate

Microbial activity can be assessed using the same experimental procedure above and using techniques to measure: (i) substrate-induced respiration (SIR); and (ii) estimation of cellulolytic enzyme activity to indicate rates of breakdown of plant material, using techniques described in Birch *et al.* (2004) (Gilligan and Reese, 1954; Miller, 1959; Anderson and Domsch, 1978).

Measured endpoint: Rates of carbon release and cellulase activity per gram of soil in the differently treated soils. Impacts on the microbial community can also be investigated using the molecular techniques described earlier.

Testable hypothesis 2: Ammonification rates will be reduced in soils with Bt-cotton residues, with consequent effects on soil fertility and subsequent crop production.

Experiment: measurement of microbial ammonification rates

Estimate the mineralization potential by the waterlogged incubation method of Waring and Bremner (1964). Add 12.5 ml of soil to 5 g of soil in an incubation jar. Seal and incubate at 40°C for 7 days. Then add 12.5ml of 4 M KCl, shake for 1 h, filter through Whatman no. 1 paper and analyse for NH_4^+–N.

Measured endpoint: Comparative rates of NH_4^+–N formation will indicate whether the presence of the Bt proteins affect rates of organic matter breakdown and subsequent mineralization.

Testable hypothesis 3: Inorganic nitrogen-transformation rates will be reduced in soils with Bt-cotton residues, with consequent effects on soil fertility and subsequent crop production.

Experiment: measurement of nitrification rates

Potential nitrification rates can be estimated by the method of Belser and Mays (1980). Amend 25 g of each soil sample with $(NH_4)_2SO_4$ and $NaClO_3$ solutions to give a final concentration of 4 mM and 15 mM, respectively. Incubate at 20°C for 48 h.

Measured endpoint: Nitrification rates are calculated from the rate of accumulation of NO_2^-–N over time. This will show if the transgenic line has a significant effect on the rate at which nitrogen becomes available to the plant.

Testable hypothesis 4: Macrofaunal activity and interactions (e.g. earthworms) will be adversely affected with a resultant decrease in plant-residue diminution and incorporation rates.

Example experiment: impact of transgenic plant material on earthworms

Methods have been developed for the standardized testing of the impact of transgenic plants on earthworms in both field soil and laboratory incubations (Zwahlen *et al.*, 2003b). Worms are introduced into containers filled with subsamples of the various soils from the lysimeters. Mortality and individual weights are recorded every 40 days over the 360-day period.

Measured endpoint: Survival and development rates of the earthworms will indicate whether the residues of the Bt cotton had an adverse effect on the growth and development of these disseminators, which has consequences for nutrient recycling and crop growth.

Modifications of this method can be used to study the effects of Bt residues and soils on other macrofaunal components, as further information on the macrofauna found in Brazilian cotton-cultivation agroecosystems becomes available.

Testable hypothesis 5: Inputs from Bt cotton will alter the survival and efficacy of soil-borne fungal and bacterial diseases of cotton, compared to the influence of the non-transformed isoline.

Experiment: assessment of the pathogenicity of soil in which Bt cotton has been cultivated

Cultivate cotton plant seedlings from a susceptible cultivar in soils in which Bt cotton and the isoline have been grown. Transplant the young cotton seedlings into five replicate pots of each soil type and grow for 16 weeks under ideal conditions in a controlled environment chamber. Then assess the relative degree of disease development.

Measured endpoint: If there is no difference in the degree of pathogen development between plants cultivated in either soil, then the Bt proteins have had no significant effect on pathogen survival in the soil.

Discussion and Suggestions for Further Development

The cultivation of Bt cotton might have significant implications for functional dynamics in soil ecosystems. Crop production is reliant on the successful recycling of plant nutrients in the soil. Such nutrient cycling is entirely dependent on microbial and macrofaunal functional activities, which in turn obtain their energy requirements from the plants. This interdependence is partly shaped by the kind of plant residues and other inputs entering the soil. Changes in the crop type, species or cultivar, whether transgenic or not, will affect the functional dynamics of the soil ecosystem. However, those changes occurring as a consequence of the cultivation of Bt crops, which express relatively large amounts of a novel protein in their tissues, may possibly be more profound. We now understand that agricultural practices such as tillage and herbicide use, and environmental factors such as sampling date and soil type, have strong effects on microbial diversity (Fig. 10.3). Studies on changes in community structure caused by transgenic plants in temperate regions have led to the conclusion that these transient changes have no lasting impact, because they have not persisted after winter; the structural composition returns to the same state as the previous spring (Dunfield and Germida, 2004). However, the situation in a tropical climate and soil may be very different and conclusions from studies in temperate regions may have only limited relevance. A consequence of major concern was what effects the active Bt toxins may have on both the microbial and macrofaunal populations in soil ecosystems. Several hazard scenarios and hypotheses to assess the impacts of Bt cotton

on soil ecosystems in Brazil were identified. The major theme in all of these was the possibility that the Bt toxins may have adverse effects on biodiversity and the functional dynamics in the soil.

Soil ecosystem populations can be described on a population constituent basis, to type, genera or species level, or on a functional basis, in which the ability to perform certain events is defined. These two population characteristics are not necessarily related; in particular, the amount of function being expressed at a particular time is most probably not related quantitatively to the numerical contribution of individuals with that ability to the total population at that time. Soil ecosystems contain tens of thousands of species, and consequently describing populations in this way is a very difficult and complex operation. An alternative approach is to define such communities by what the community and its parts do, i.e. by defining functional properties. Functions generally can be associated with many individual species, and so provide a manageable way of describing populations. Biodiversity needs to be considered here. As only a few of the many species capable of a particular function are required at any particular time to maintain that function, functional measurements alone may obscure a change in the variety of different individual species with that characteristic in the community. Although a definitive definition of the extent to which such reductions in biodiversity can be tolerated before system sustainability is compromised is difficult to determine, it is thought best practice to sustain as high a level of biodiversity in the system as possible.

We recommend that the impacts of Bt cotton on the soil ecosystem be assessed by a combination of both functional and structural assays. As key microbial and macrofaunal functional groups are responsive to plant inputs and cultural conditions that are involved in nutrient cycling, and so soil fertility, they have great potential for use in determining any impacts on soil ecosystems. The effects of transgenic plants on soil ecosystems will therefore be best studied by the application of several basic and well-proven microbial and macrofaunal activity assays. Experimental protocols were devised to assess Bt toxin persistence in soils, changes in microbial population biodiversity, and effects on plant residue decomposition and subsequent nitrogen-transformation rates. The measured endpoints from these will enable crop-management decisions that aim towards sustainable and ecologically sound Bt-cotton cultivation in Brazil.

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